INFLUENCE OF SODUIM OXAMATE ON OIL CONTENT IN CANOLA LEAVES

A Thesis

by

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MASTER OF SCIENCE

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ABSTRACT

Biofuels that share similarities to petro-diesel regarding combustion properties have received renewed attention. Triacylglycerols (TAG) comprise the main compound in plant oil seed crops and animal fats that is used for biodiesel. In senescent leaves, TAGs are the most abundant pool of lipids. TAGs contain higher levels of fatty acids than are found in chloroplast membranes. Synthesis of glucose from non-carbohydrate carbon substrates occurs via gluconeogenesis from the oxidation of TAGs into glucose, pyruvate, lactate, and glycerol. This study examines the effect gluconeogenesis inhibitors, such as sodium oxamate, could play in increasing oil accumulation in plants. Also, this study tests the efficacy of sodium oxamate in increasing TAG and membrane lipids in *Brassica napus*. Several concentrations of sodium oxamate (control, (water with surfactant), 0.0005 mM, 0.005 mM, 0.05 mM, 0.5 mM) were applied to plant leaves. Oil was extracted by using chloroform/methanol (2:1) in accelerated solvent extraction. No oil bodies were observed in the leaves from the vegetative to the reproductive stages for each treated plant. Extracted oil from each plant was separated using thin layer chromatography (TLC) to determine if changes in lipid composition occurred in leaves. We concluded that sodium oxamate did not affect the oil accumulation in leaves of *B.napus* under the conditions tested. Further studies should be conducted with alternate species and varying surfactants to understand the role that sodium oxamate plays in altering gluconeogenesis driven oil content.

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Finally, thanks to my mother and father for their encouragement, and to my wife for her patience and love.

NOMENCLATURE

ASE= Accelerated solvent extraction

ANOVA= Analysis of variance

PC = Pyruvate carboxylase

TAG = Triacylglyceride

TLC= Thin layer chromatography

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CHAPTER I

INTRODUCTION

According to the USDOE (2007) and USDA (2007), the total plant oil production across the world is around 120 million tons, which would only account for about 80% of the total diesel needs of the United States. Most of the world energy supply is from fossil fuel (i.e., oil, natural gas and coal). When considering production and consumption rates, reserves of fossil fuel could last roughly 700 years (Goldeemberg & Johansson, 2004; Goldeemberg, 2007). Because of the limited supply of fossil fuels, the growing concern of energy security, and the importance of considering climate change, there is increasing interest in renewable energy sources such as biofuels (Marica, 2009).

Biofuels that share similarities to petro-diesel and combustion properties are receiving renewed attention. Despite this attention, several limiting factors related to plant oil production include limited land, water, and competition to food crops. These factors result in decreased plant oil production overall. However, some of these limitations may be overcome by using a substitute resource for plant oil, or by improving the amount of oil produced by plants in non-seed tissues (Durret, Benning, & Ohlrogge, 2008). Triacylglycerols (TAGs) stored in seeds are not only the most common storage form of lipids in plants, but they are also the most important form (Appelquist, 1975; Lin & Oliver, 2008). Plant oil primarily from seeds, but also from leaves, are used for food and feed production, industrial chemical purposes, and are chemically similar to petroleum fuels (Durret, Benning, & Ohlrogge, 2008; Patel & Crank, 2006).

It has long been demonstrated that sucrose is a hydrolytic product of glucose and fructose, which are the first step of glycolysis and the end step of gluconeogenesis in higher plants (Sung, Xu, Galloway, & Black, 1988). Plant glycolysis takes place both in the cytosol and plastid (Plaxton, 1996). In glycolysis, pyruvate and acetyl CoA are formed by the action of pyruvate kinase from phosphoenolpyruvate (PEP) (Rawsthorne, 2002). Pyruvate dehydrogenase respectively, Acetyl CoA is the building block for fatty acids (FAs) stored as TAG and also the oxidation product of (FAs) through gluconeogenesis.Synthesis of glucose from non-carbohydrate carbon substrates occurs via gluconeogenesis from the oxidation of TAGs into acetyl CoA. While the gluconeogenesis pathway in plants. Partially differs from other organisms, it is still the primary regulator of fatty acid metabolism. And as such, its inhibition could be a rationale target for increasing oil accumulation in plants, and possibly increasing oil accumulation in leaves.

The long-term goal of this project is to study the effect gluconeogenesis inhibitors, such as sodium oxamate, could play in increasing oil accumulation in plants. The *primary objective of this study* is to test the efficacy of sodium oxamate in increasing TAG and membrane lipids in *Brassica napus*. The *central hypothesis of this thesis* is that there is a correlation between the effect of sodium oxamate treatment with some enzymes in gluconeogenesis and the Krebs cycle, and that the correct molar treatment will inhibit fatty acid oxidation in leaves and increase oil accumulation. This hypothesis will be tested using the following objectives:

***Objective 1*: Determine the efficacy of sodium oxamate for increasing TAG and membrane lipids in *Brassica napus*.**

***Objective 1.1:* Determine the optimal dose rate of the gluconeogenesis inhibitor, sodium oxamate, and timing for maximal foliar oil accumulation.**

***Objective 1.2*: Determine if sodium oxamate increases oil body accumulation in the leaves of *B. napus*.**

***Objective 1.3:* Determine the total leaf oil content following various treatments of sodium oxamate by using accelerated solvent extraction.**

***Objective 2*: Determine if sodium oxamate alters the accumulation of alternate pools of oil in TAGs, FA's, and others.**

CHAPTER II

LITERATURE REVIEW

The biodiesel industry needs an eco-friendly diesel obtained from renewable resources such as plant oils and animal fats that can then be used in cars, trucks, and home heating oil units (Bajpai & Tyagi, 2006). Triacylglycerols (TAGs) comprise the main storage oil pool found in plant seeds and in animal fats that are used for biodiesel. Biodiesel has advantages over traditional diesel, such as lower carbon monoxide emission compared to conventional diesel, and the fact that it is a renewable resource that facilitates faster biodegradation (Durret, Benning, & Ohlrogge, 2008). In addition, biodiesel is similar to fossil fuel in chemical and physical properties, and has good lubricity (Bajpai & Tyagi, 2006). Biodiesel is not only preferable to conventional diesel, but also to ethanol due to several other key advantages: first, the energy content per volume is 25% higher in biodiesel than ethanol; second, corrosion of pipelines can occur when ethanol is used; and third, biodiesel requires significantly less energy to extract to convert hydrocarbons into ethanol (Durret, Benning, & Ohlrogge, 2008).

Biodiesel can be obtained from animals, plants, and microorganisms. For example, TAG is found in milk in the range C26 - C54 with fatty acid from 4:0 to 24:0 (Gresti, Bugaut, Maniongui, & Bezard, 1993). Plants store TAG in their seeds and in some cases in their leaves. During senescence, and prior to flowering, TAGs have been found to accumulate in leaves (Lin & Oliver, 2008). Sakaki et al. (1990) found that "monogalactosyldiacylglycerol (MGDG) converted to 1, 2 diacylglycerol and acylated

with 18:3 to TAG in ozone-fumigated spinach leaves" (page 766). The TAGs in leaves contain the same fatty acid as is found in the leaves of some plants (Lin & Oliver 2008). In algae, TAG biosynthesis can occur via the glycerol pathway (Ratledge, 1988). Furthermore, yeast and fungi lipids contain TAG and fatty acids similar to those found in vegetable oil (Blagovi et al., 2001; Gill, Hall, & Ratledge, 1977).

Biodiesel TAG in leaves

Kaup, Froese, and Thompson (2002) reported that biodiesel TAG is found in the rosette leaves of *Arabidopsis*, and that TAGs are also the most abundant pool of lipids in senescent leaves. Moreover, they found that the TAGs were at higher levels of fatty acids than found in chloroplast membranes. Other researchers found that, "the leaves of some plants species contain higher concentrations of TAG, and the fatty acids in the TAG are distinct from the fatty acids in membranes and more closely relate to the TAG found in their seeds" (Lin & Oliver, 2008, p.236). The physiological role of TAG in leaves is not completely understood, yet it may be metabolic or for temporary storage. In some species, oil bodies containing TAGs occur in the mesophyll cell of leaves (Lersten et al., 2006). Lersten and colleagues (2006) suggest that oil bodies could serve as a potential new source of TAG oil for increased production of biodiesel.

Gluconeogenesis

Gluconeogenesis is the synthesis of glucose from non-carbohydrate carbon substrates such as lipid, pyruvate, lactate, glycerol, and glucogenic amino acids. The fatty acid group is removed by lipases and various lipid droplet-associated proteins. Moreover, it could be used in endoplasmic reticulum membrane lipid synthesis or

transported into peroxisomes and degraded by β-oxidation (Graham, 2008). In seedling and in senescing leaves, the glyoxylate cycle enzymes participate in the conversion of FA to organic acid, which is exported to the cytosol and mitochondria, and which supports the gluconeogenesis pathway (Chapman, 2013). Pyruvate carboxylase (PC) is a regulatory metabolic enzyme that is responsible for renewing the intermediates of the Krebs cycle and stimulating the first step in gluconeogenesis (Wallace, 1985). Acetyl-CoA is a positive effector of PC that is produced from fatty acid oxidation, and converts to oxaloacetate for subsequent conversion to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK; Pelley & Goljan, 2010; Fig 1). PEPCK occurs as distinct isoforms, and will the mitochondria isoform converting oxaloacetate to PEP (Hanson & Patel, 1994).

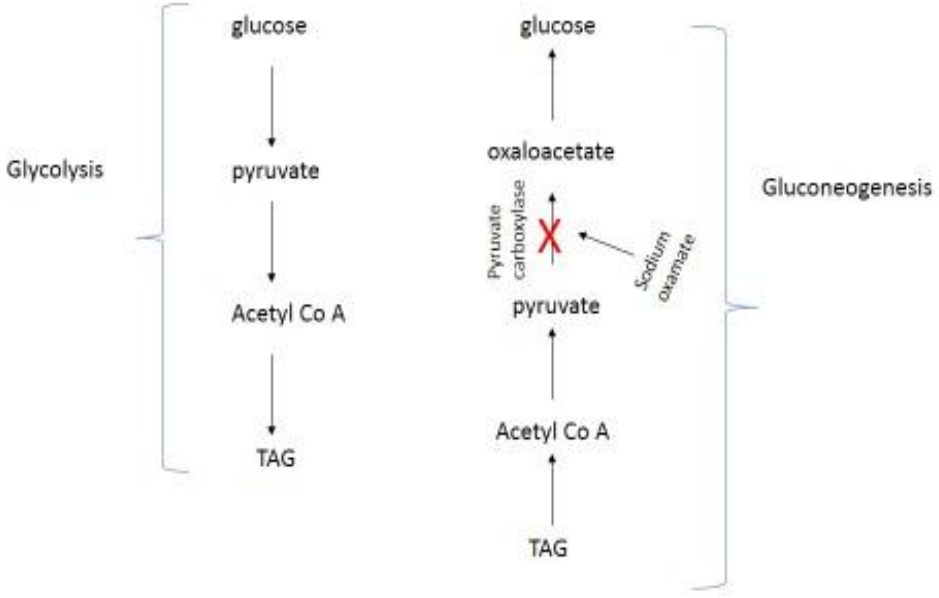

Figure 1 Glycolysis and gluconeogenesis pathways with gluconeogenesis inhibitor (sodium oxamate) inhibits pyruvate carboxylase.

Gluconeogenesis inhibitor

Oxamate is known to inhibit lactate dehydrogenase (Wilkinson & Walter, 1972). The chemical structure of oxamate is an analogue of pyruvate that binds in the carboxyl transferase (CT) domain of pyruvate carboxylase, and is followed by the movement of the tethered biotin from the biotin carboxylase (BC) domain to the CT domain, which is then induced (Goodall, Baldwin, Wallace, & Keech, 1981; Fig 2). Martin-Requero, Ayuso, and Parrilla (1986) evaluated the physiological relevance of oxamate inhibition of PC on the rates of hepatic gluconeogenesis. Oxamate caused a decrease in the concentrations of all metabolites after pyruvate, which indicated that the inhibitory effect on gluconeogenesis was due, in part, to the inhibition of PC. Oxamate acts as a competitive inhibitor of gluconeogenesis at less than 0.4 mM of pyruvate (Martin-Requero, Ayuso, & Parrilla, 1986).

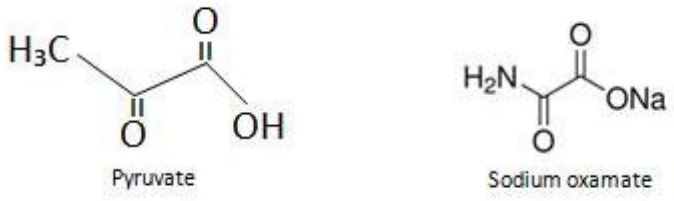

Figure 2 The chemical structure of pyruvate and sodium oxamate.

CHAPTER III

MATERIALS AND METHODS

Plants

One cultivar of spring canola was chosen (e.g. treated sumner). Plants were grown for 3 to 4 months in a greenhouse in 7.6 -L pots, each with two plants.

At three months prior to flowering, plants were treated with various concentrations of sodium oxamate (0.5 mM, 0.05 mM, and 0.005 mM, 0.0005 mM, control with surfactant, and control). A spray chamber was used in this project to spray sodium oxamate with following condition: 140.25 L/ha, 4.8 Km/h, and 50 cm nozzle spacing. The surfactant Agidex (0.25%) was added to the sodium oxamate solution.

Prepare tissue

After 24, 48, 72, and 96 hours from the spraying of chemical material, the leaves were harvested and preserved at -80 C. and the sample was freeze dried for accelerated solvent extraction of leaf oils.

One plant from each pot was collected for sectioning purposes. Sections were made from young leaves, middle leaves, older leaves, and stems. The harvested leaves were stored in a Ziploc bag at C prior to sectioning. Sectioning was completed 1 to 3 days post collection.

A free-hand sectioning and staining method using Sudan IV dye was performed as follows per Lin & Oliver (2008): Sudan IV was prepared by adding 1 g of Sudan IV to 500 ml of absolute ethanol to make a stock solution. Then, the diluted solution of 62.5 ml of absolute alcohol was mixed with 50 ml of distilled water. Next, 87.5 ml of the

stock solution of Sudan IV was added to the diluted solution to form the final dye. Free-hand sectioning was examined using light microscopy under 10x or 25x magnification. Two-seven digital images were taken for each positively stained tissue sectioned. Positive stained tissue clearly showed oil body, and the percent leaf oil body area of each image was calculated.

Accelerated solvent extraction (ASE)

This procedure is based on the methods used by Luthria, Vinjamoori, Noel, and Ezzell (2004), Dionex (2004), and Dionex (2006) to extract lipids from leaves. Leaf tissue was first dried in a freeze dryer and then ground with a coffee grinder. One gram of ground tissue was used for the ASE of each sample. For sample loading and lipid collection, an 11-ml extraction cell with a cellulose disk at the end of the cell was loaded with the sample mixed with the dispersing agent hydromatrix. Polar chloroform with methanol 2:1 was used as extraction solvent in this study.

The ASE conditions were based on Dionex (2004) and Dionex (2006) as follows: The ASE was set to (6.67MPa) at 105° C for 5 min with a 100% flush volume and 60 sec purge time for 1 static cycle. From the collection vials, the extraction solution was pipetted into five pre-weighted 1.8 ml vials at a volume of 1.5 ml for each vial. To evaporate solvent, vials were placed in a Speed Vac. Vials were then reweighed, and the total oil extracted from the cells were calculated by equalizing the total weight of lipid from each 1.5 ml sample to the total solution extracted, which ranged from 15 to 22 ml. Thus, the average total lipid for each treatment was calculated.

Statistical analysis

The data was analyzed using SAS version 9.2 (SAS Institute Inc., 2008). An ANOVA factorial test was chosen to run the data because we have two factors: time and concentration. The time varied as follows: 24h, 48h, 72h, and 96h. Concentrations also varied: 0.5 mM, 0.05 mM, 0.005 mM, 0.0005 mM, control with surfactant, and control. Pots and leaves are used as reps. Moreover, one-way, and two-way tests were used to study the interaction between factors.

Thin layer chromatography

This method was adapted from Christiansen et al. (2007). Thin layer chromatography was used separate extracted lipid. The lipid sample was dissolved in chloroform and spotted onto a 20x20 cm analytical, normal phase, silica gel TLC plate (Sigma Chemicals, St. Louis, MO). The lipid standards of monoglycerides, diglycerides, triglycerides, cholesterol, free fatty acids, alcohol, aldehydes, and tocopherol were also spotted on the TLC plate. The plate was developed in a solvent system of hexane, diethyl ether, and acetic acid (85:15:2) for one hour until the solvent reached up to four centimeters below the top of the plate. After the plate dried, oil bands were stained with copper sulfate (II) (20 g of cupric sulfate in 200 ml of 8 % phosphoric acid) and then the bands were charred at 165° C for one hr. The bands turned darker after charring. Imagej was used to quantity oil in samples.

CHAPTER IV

RESULTS AND DISCUSSION

Leaf oil body sectioning

Figures 3 through 14 show images from leaf sections of each treated plant. Figures are divided into three leaf ages: young, middle, and old leaves, and inhibitor concentrations. All the figures indicate that there are no oil bodies observed in the leaves.

No oil bodies were found in the leaves, which is a reasonable result due to the findings of Lersten et al. (2006) which indicated that Brassicales did not have oil bodies in leaf mesophyll cells. Moreover, from our section results, leaf location and concentration did not affect oil accumulation in oil body, which is consistent with our results and explains why the leaves do not contain oil bodies. Additionally, over time no oil body was observed. However, the leaf lipid was increased in old leaves by the time when we used 0.0005 mM sodium oxamate. This increasing could be explained by Dahlqvist et al. (2000), who suggested for DGAT, TAGs from phospholipid:diacylglycerol acyltransferase (PDAT) seem to arise from activity associated with plant cell membranes.

Moreover, Murphy, Cummins, and Kang (1989) found that the synthesis of lipids did not occur until the third week after flowering, which is consistent with our results and explains why the leaves do not contain oil bodies. This could suggest that either oil body moves to form oil in seeds, or there is no oil body in the brassica leaf. Murphy and Parker (1984) found that the oleosomes lipid changed 40 days after anthesis.

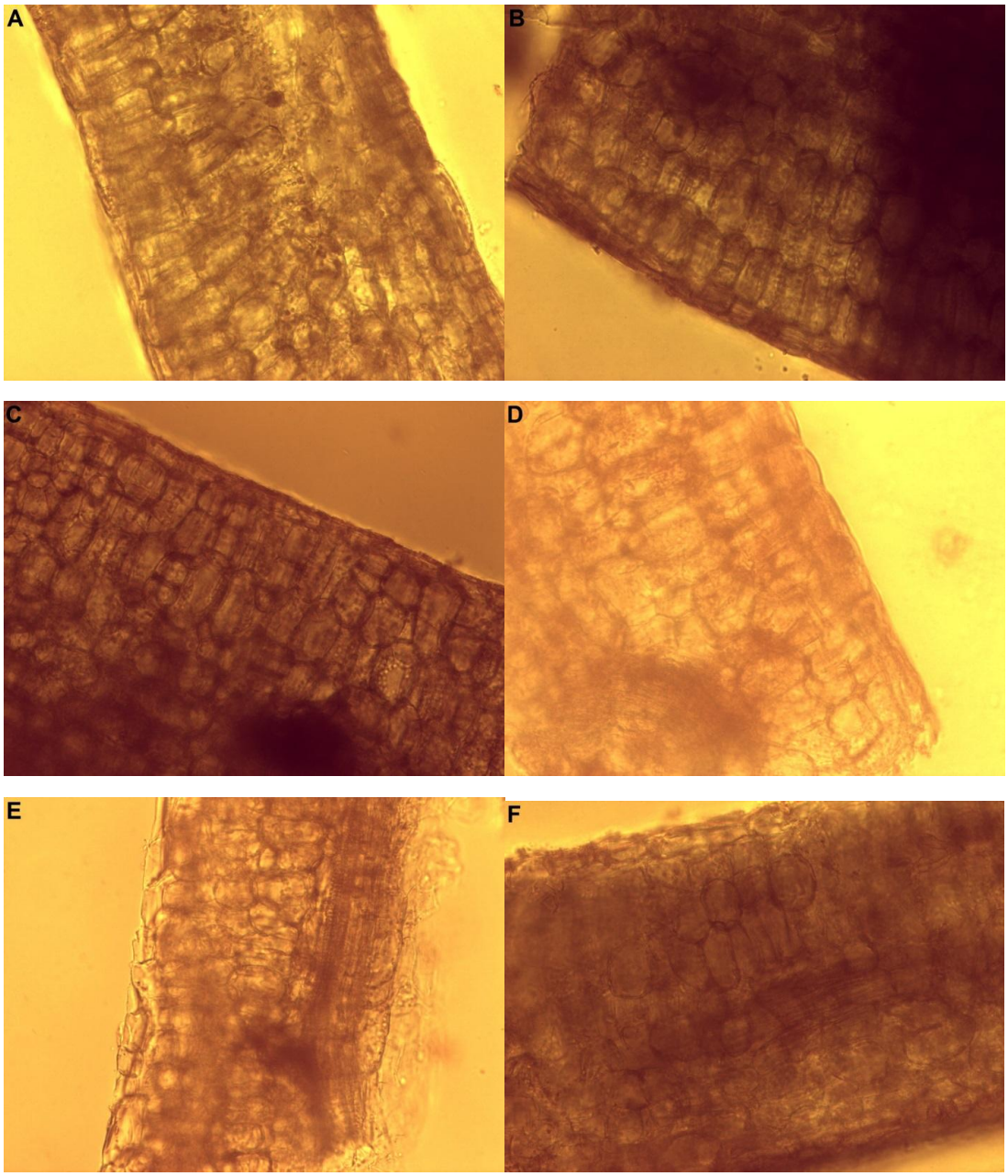

Figure 3. Stained leaf sections of treated with sodium oxamate plants 24h young leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

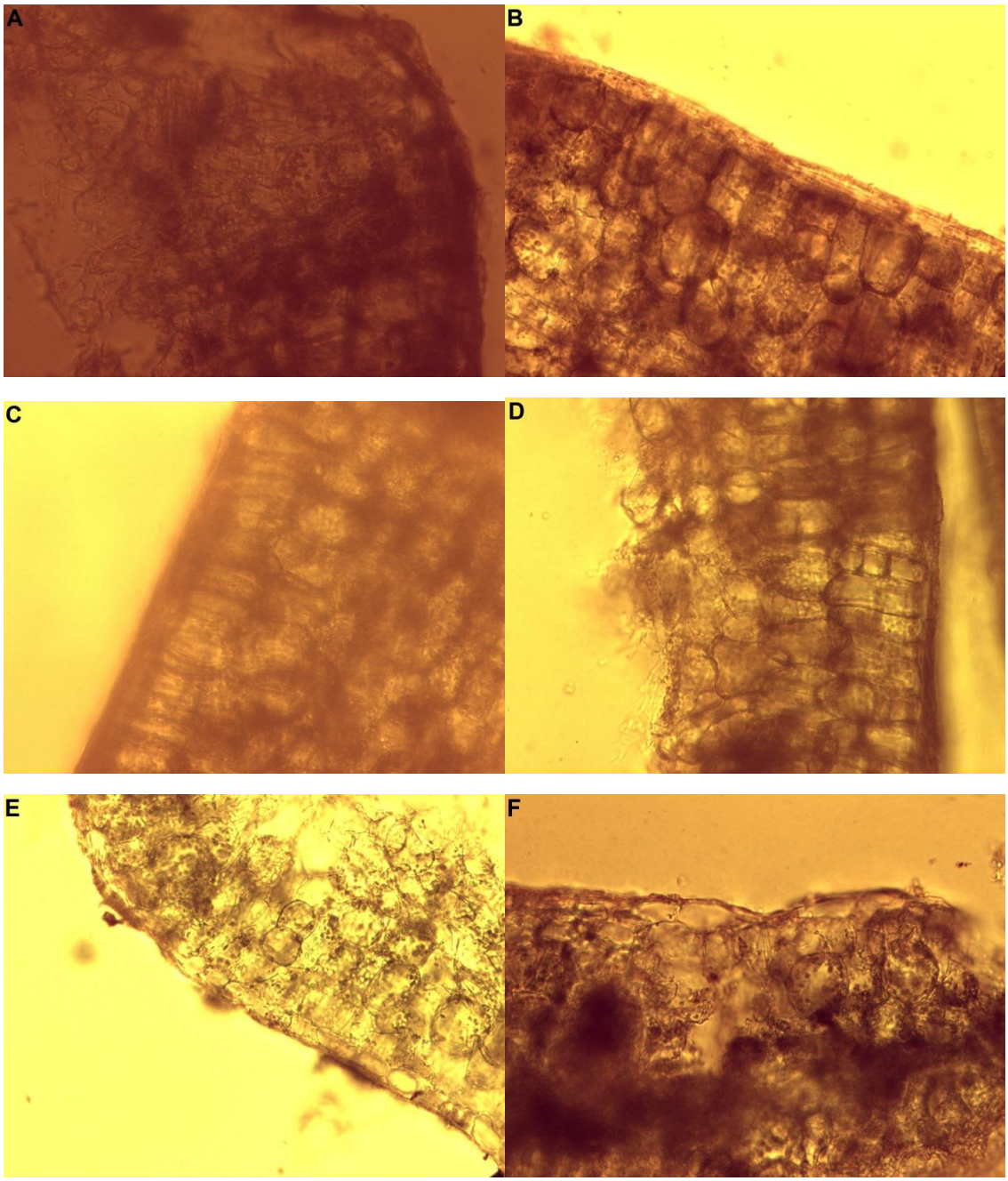

Figure 4 . Stained leaf sections of treated with sodium oxamate plants 24h middle leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

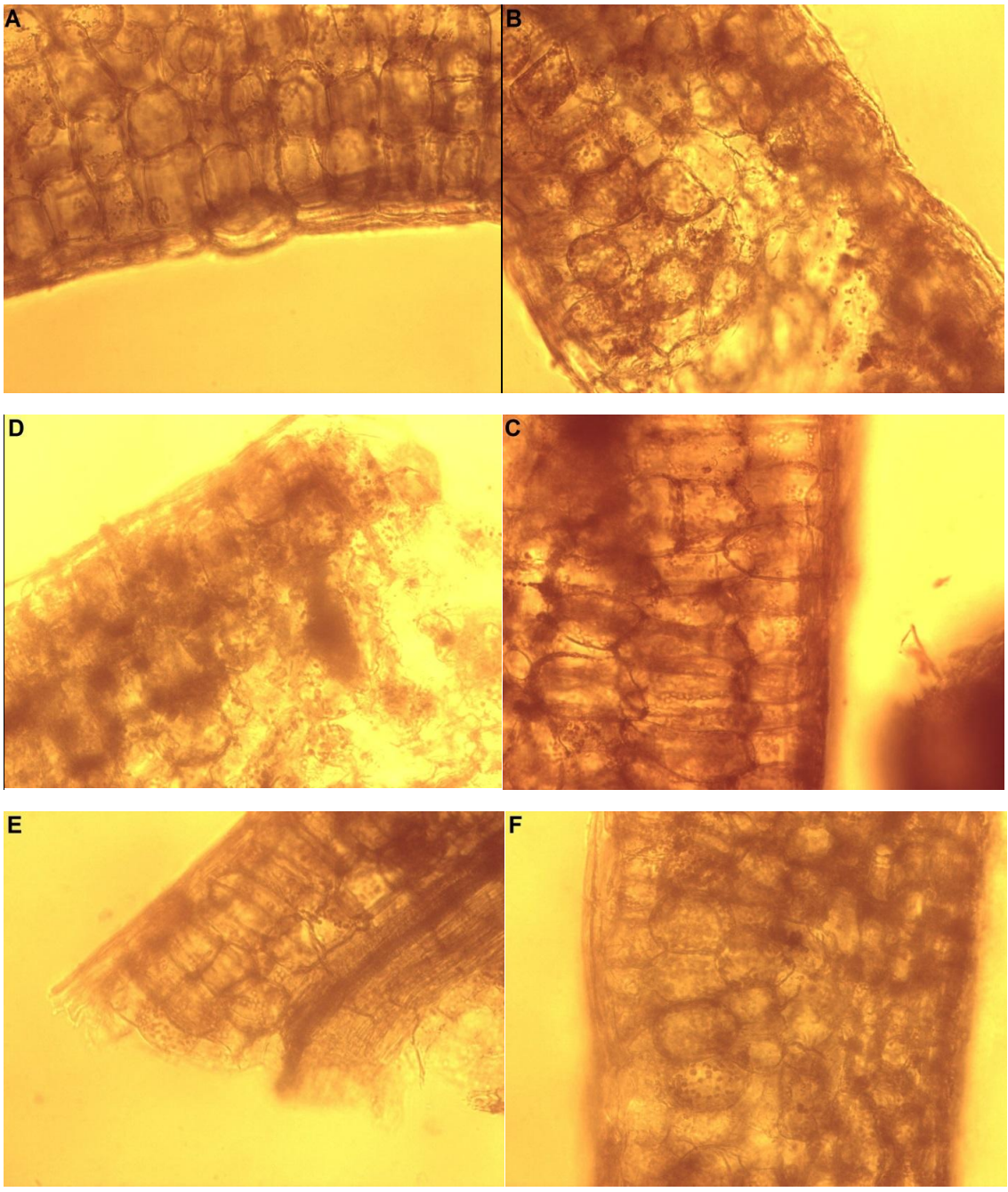

Figure 5. Stained leaf sections of treated with sodium oxamate plants 24h old leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

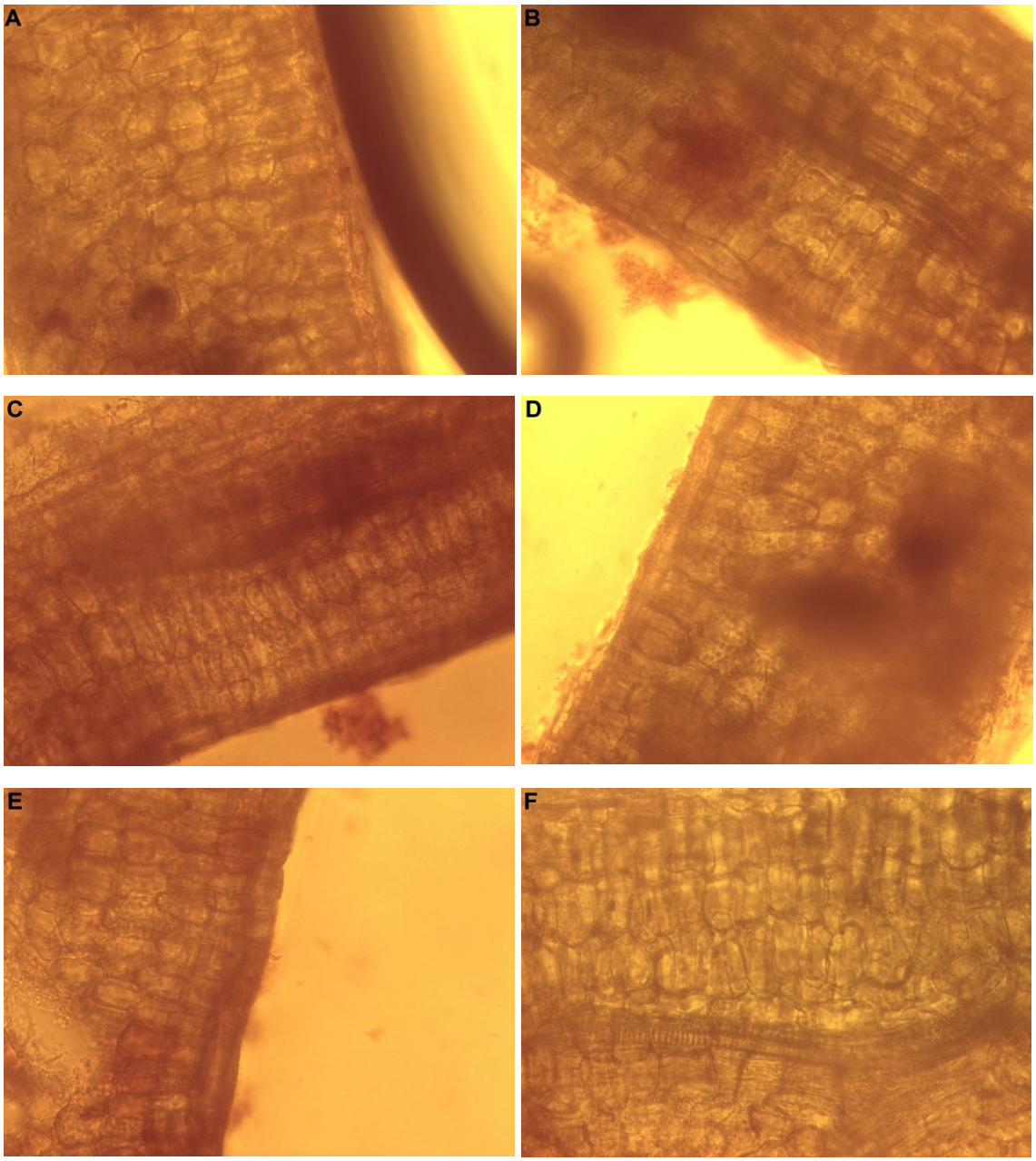

Figure 6. Stained leaf sections of treated with sodium oxamate plants 48h young leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

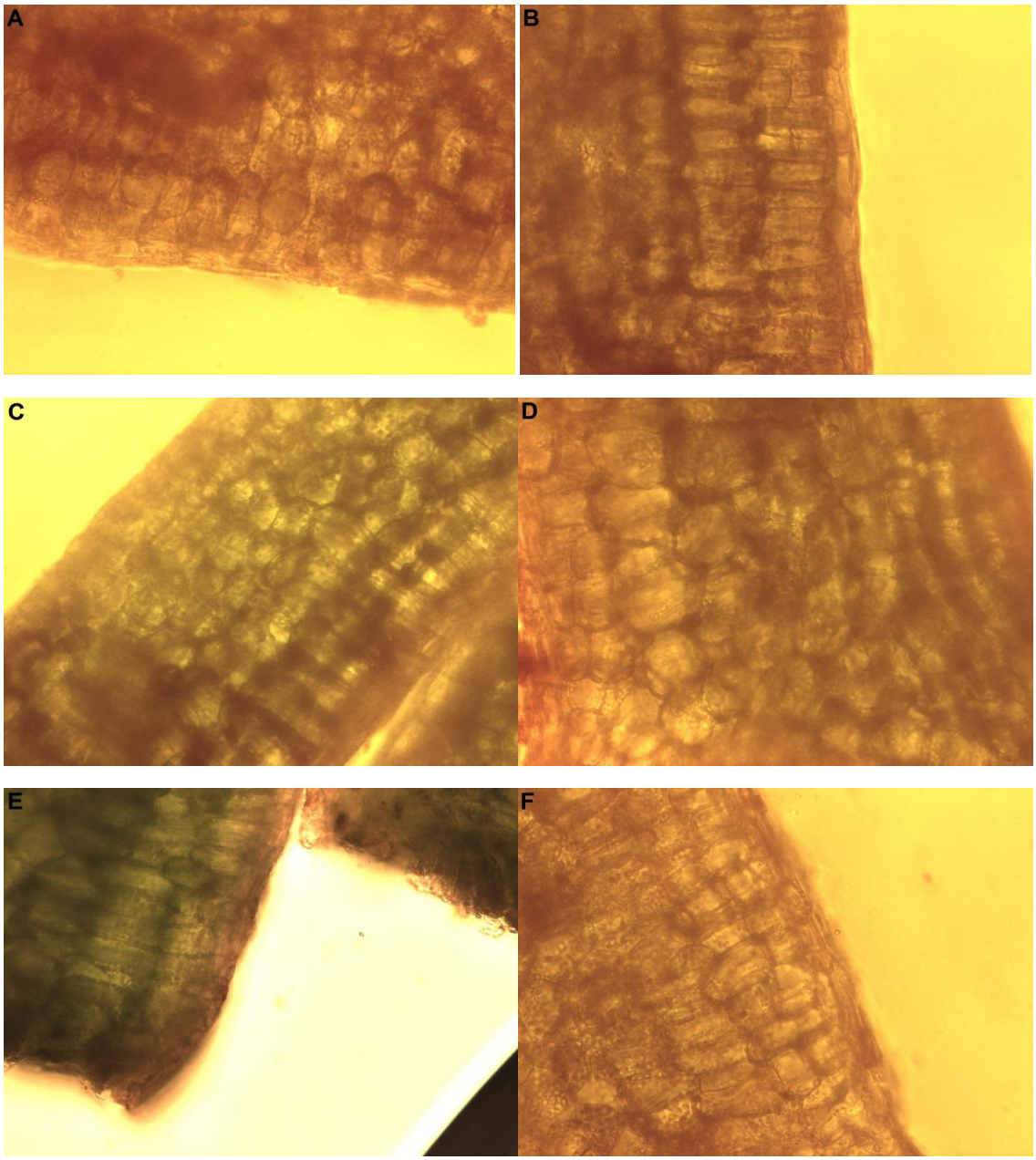

Figure 7. Stained leaf sections of treated with sodium oxamate plants 48h middle leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

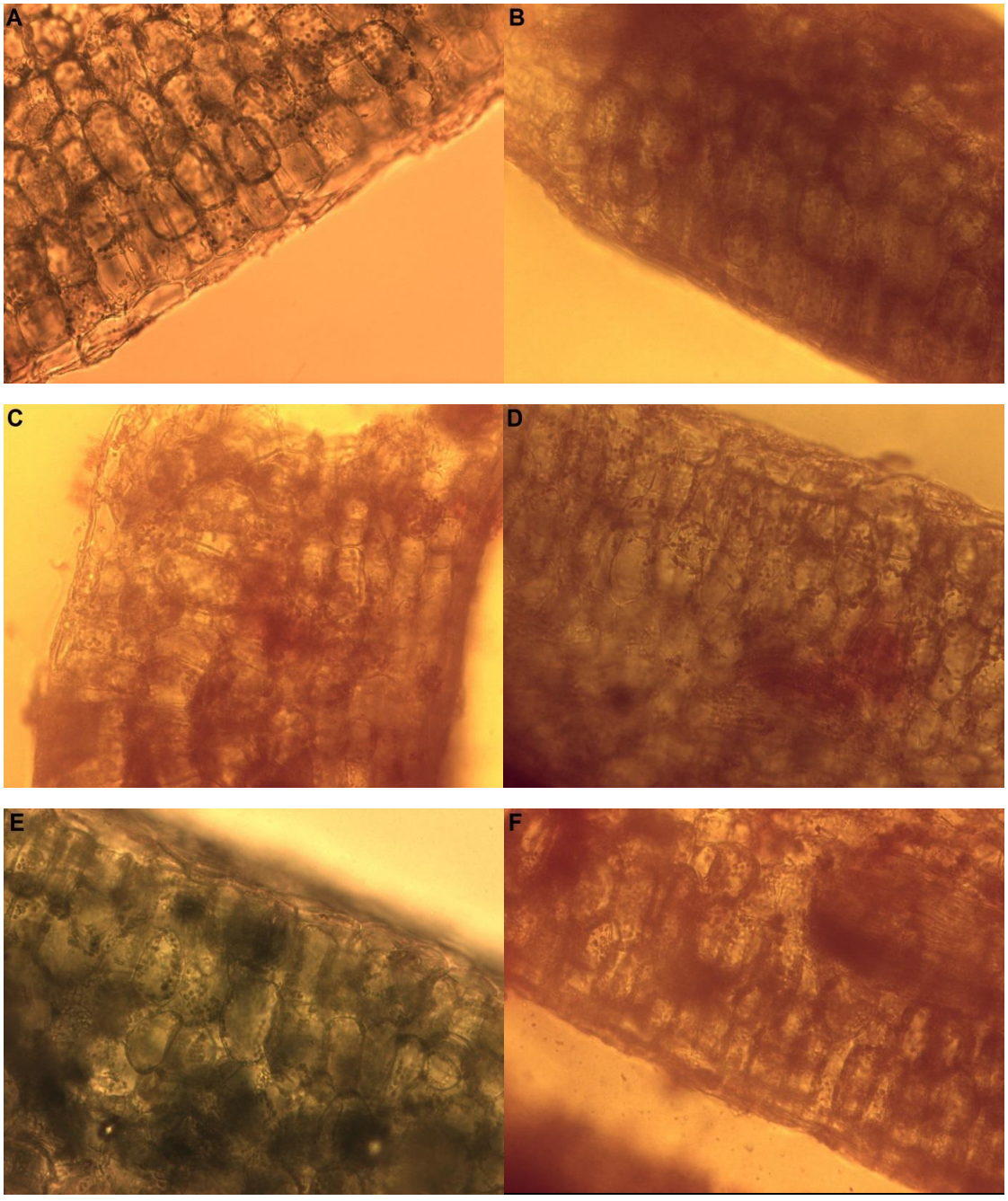

Figure 8. Stained leaf sections of treated with sodium oxamate plants 48h old leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

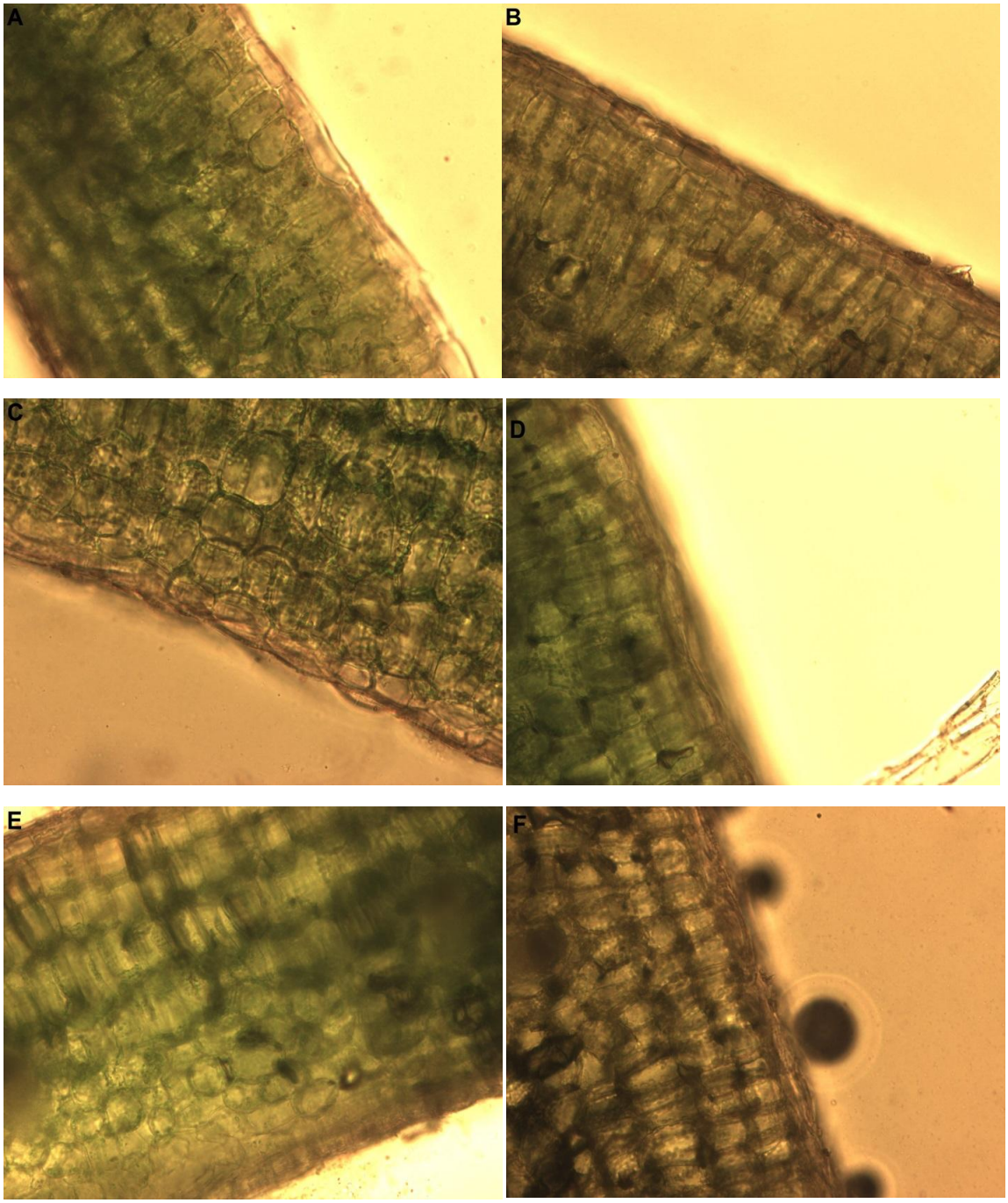

Figure 9. Stained leaf sections of treated with sodium oxamate plants 72h young leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

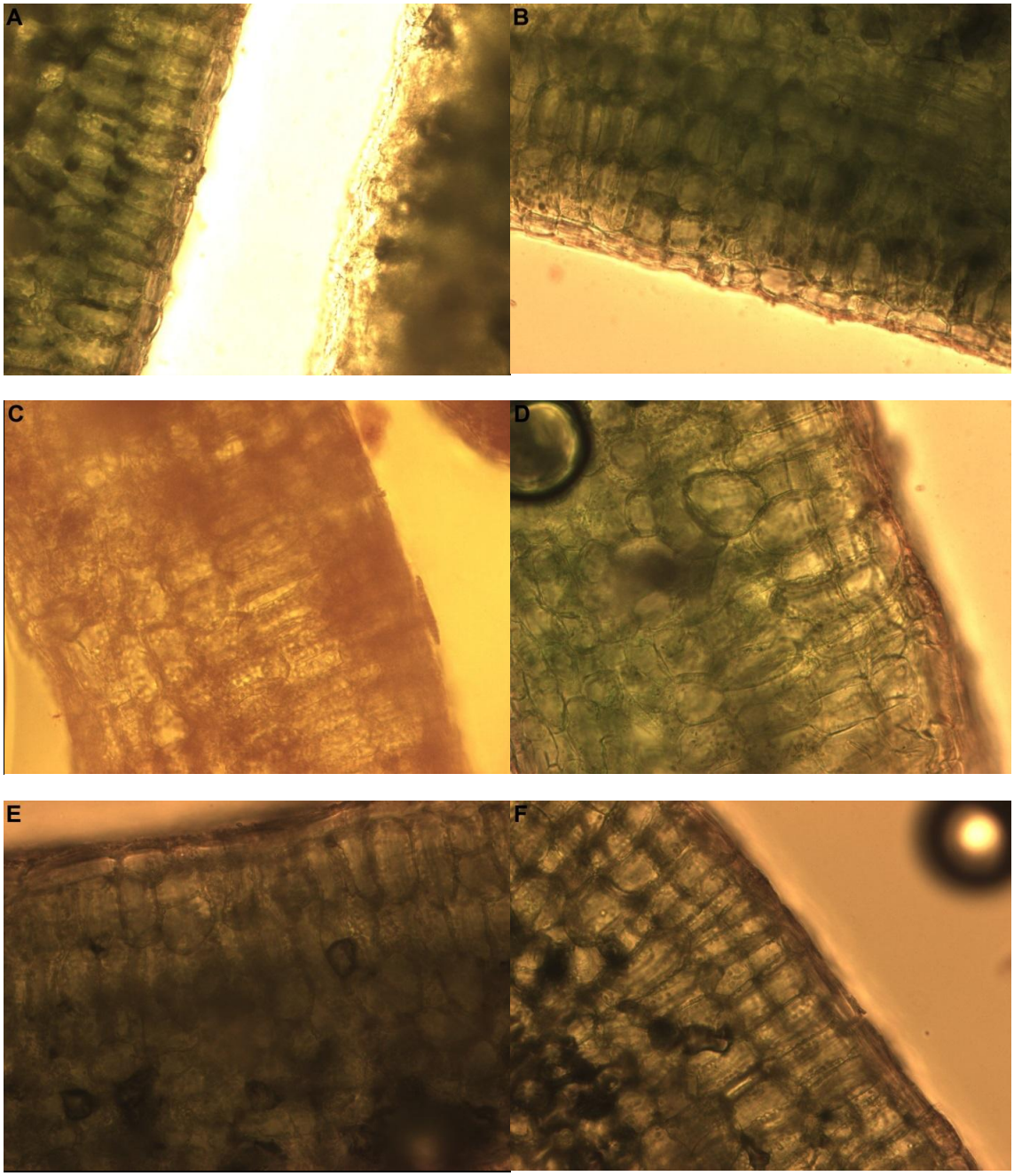

Figure 10. Stained leaf sections of treated with sodium oxamte plants 72h middle leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

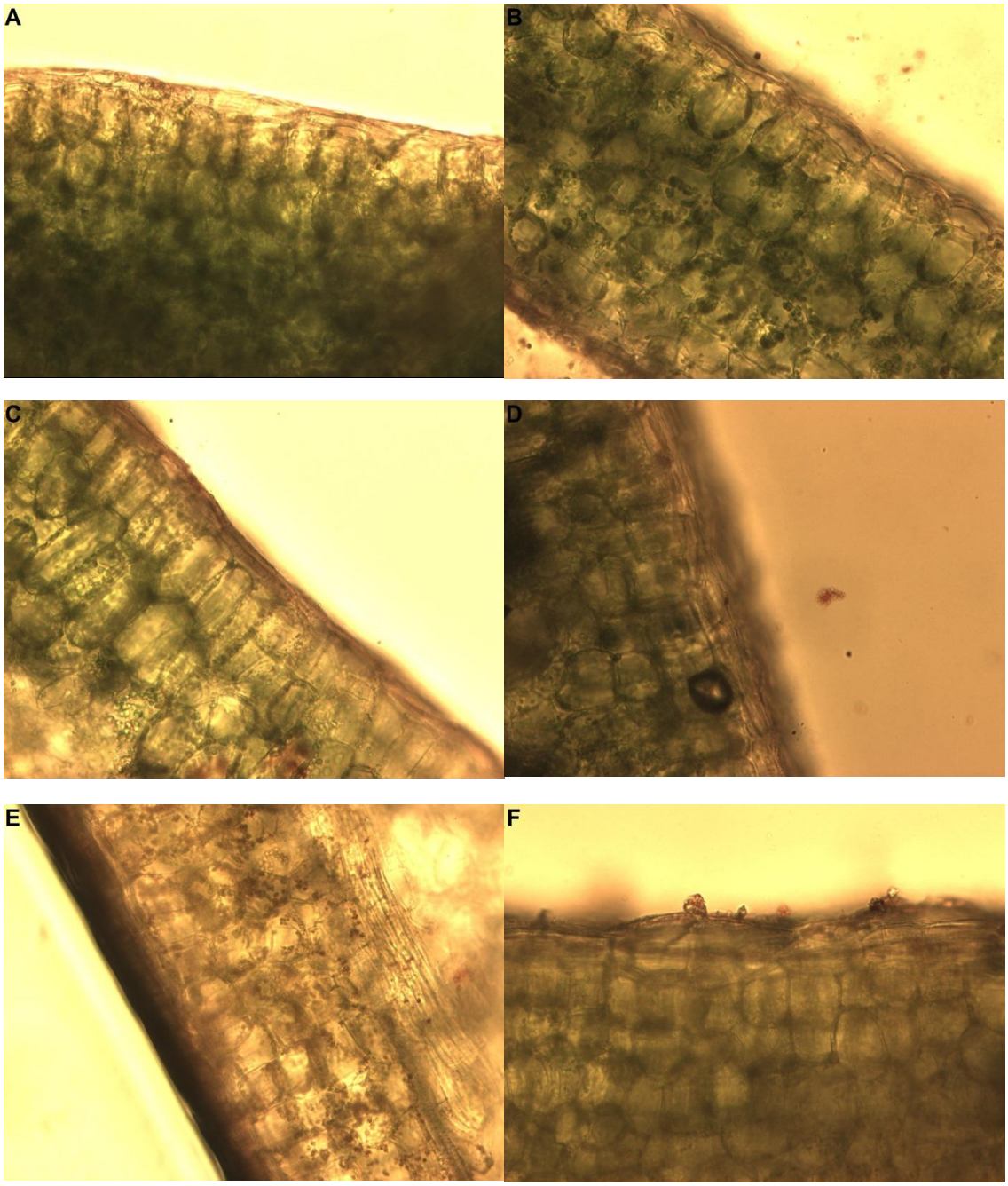

Figure 11. Stained leaf sections of treated with sodium oxamate plants 72h old leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

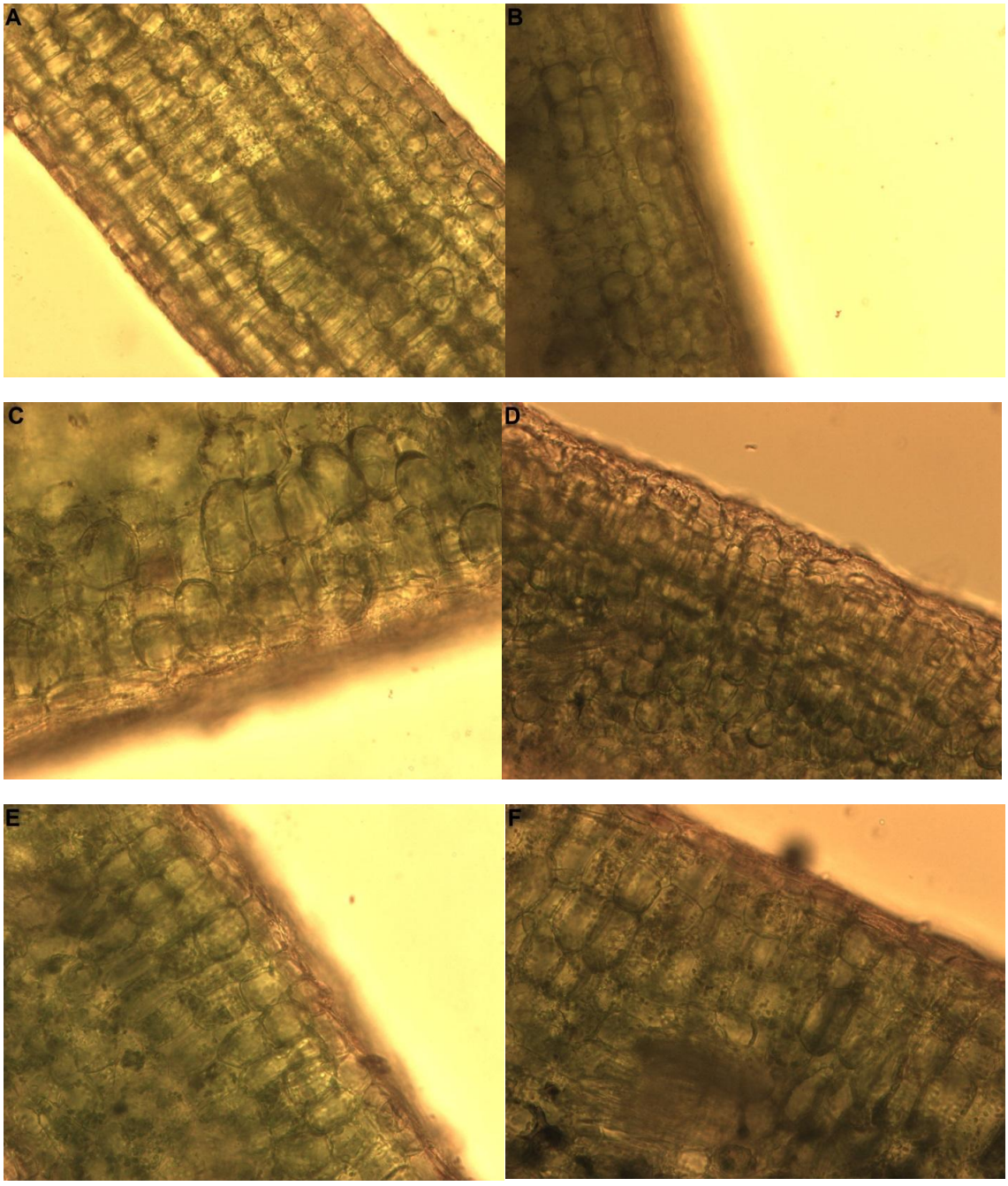

Figure 12. Stained leaf sections of treated with sodium oxamate plants 96h young leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

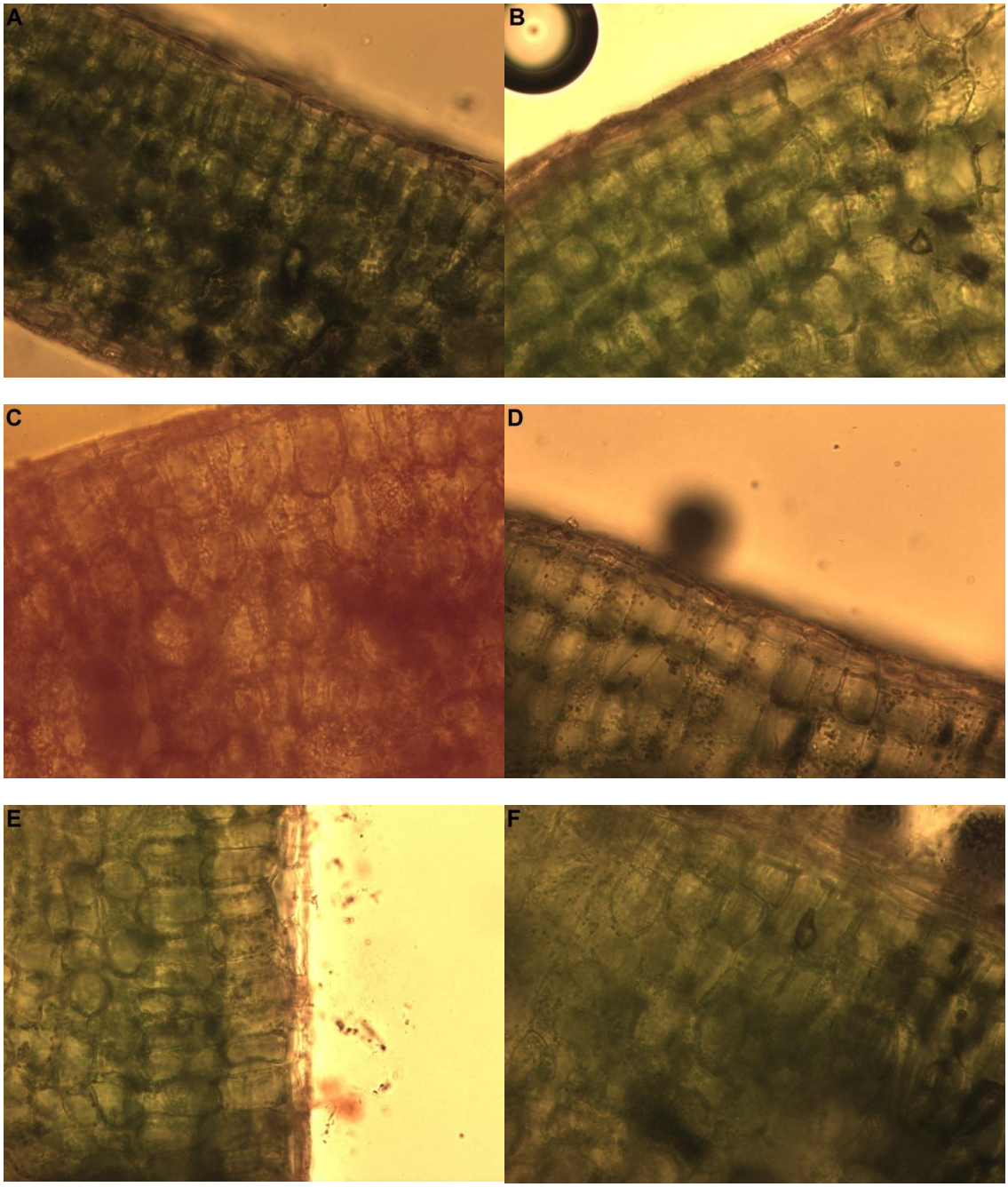

Figure 13. Stained leaf sections of treated with sodium oxamate plants 96h middle leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification 25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

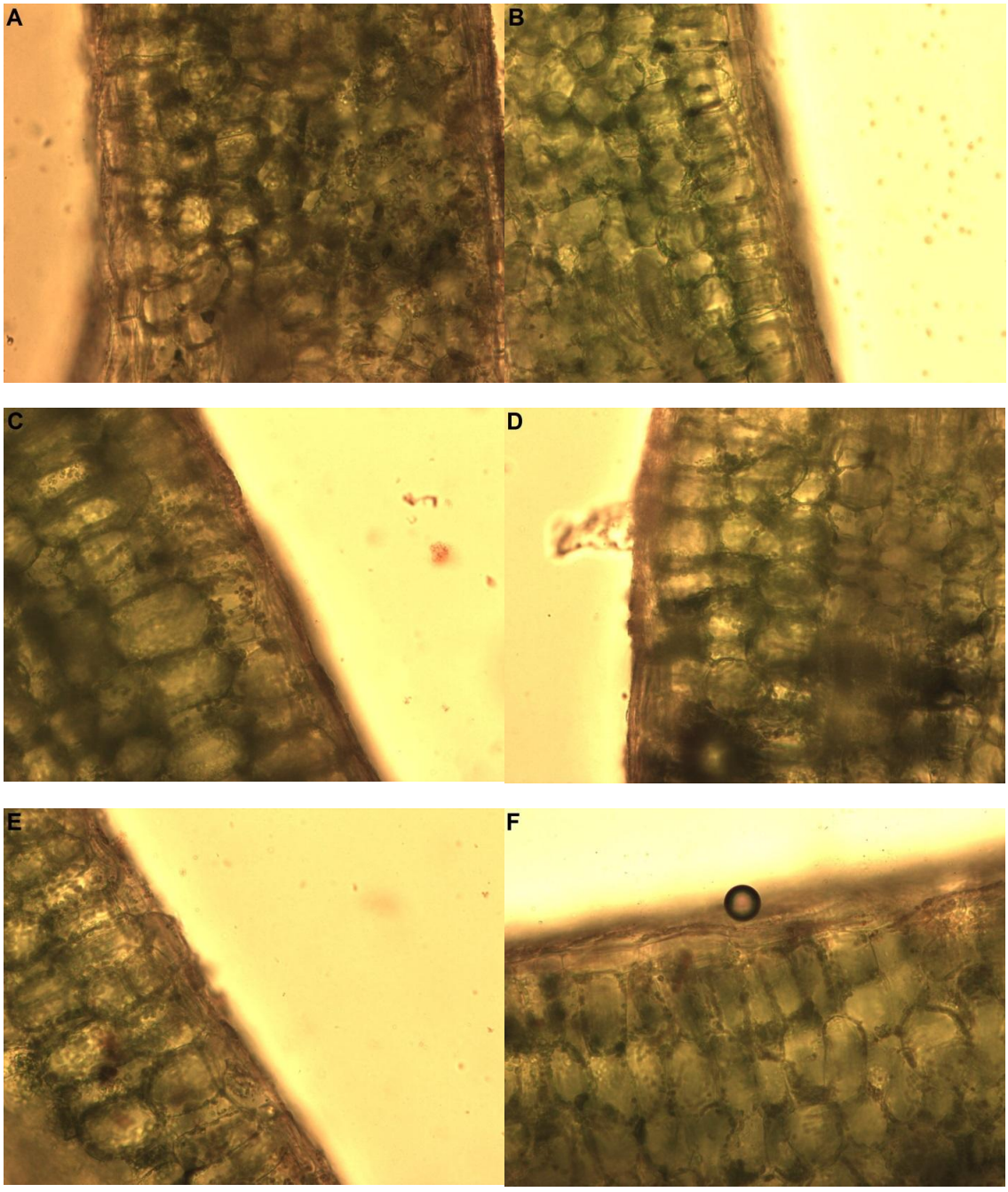

Figure 14. Stained leaf sections of treated with sodium oxamate plants 96h old leaves. Leaves were hand-sectioned and stained with Sudan IV dye. Magnification25X. These are treated plants shown are: A, control B, water with surfactant C, 0.0005 mM D, 0.005 mM E, 0.05 mM F, 0.5 mM.

Rothe (2010) found that oil body of *Cynara cardunculus* decreased over developmental time (days after planted), it could explain oil bodies decreased over the time in our study before flowering.

Lipid extraction

The results show that the lipid percentage ranged from 5% to 19% oil. The average lipids extracted by the chloroform/methanol (2:1, v/v) from greatest to least among leaf location were middle (18%), young (16%), and old (14%) (Tables 1,2,and 3). Moreover, in old leaves, the 0.0005 mM sodium oxamate was increased by the time. Oil extraction did not show any difference between the treated plants. Also, leaf location and concentration interaction as factors did not affect lipid content levels as shown in the graphs. The absence of oil bodies in the leaves could explain this result.

The interaction between time and concentration was observed in affecting the amount of extracted lipid from leaves. Also, this interaction suggests that the amount of extracted lipid changes over time in each treated plant.

Kaup, Froese, and Thompson (2002) studied the lipid composition of leaves over the time in *Arabidopsis*. They found that in senescence leaves, the concentrations of DAGs, free fatty acids, TAGs, and wax esters were higher over polar lipids. This supports our result that lipid composition in leaves changes over time.

Rothe (2010) reported that *Asclepias incarnata* contains more oil bodies in mesophyll cells and it increased over developmental time (days after planted). Sodium oxamate may increase leaf lipid in this specie.

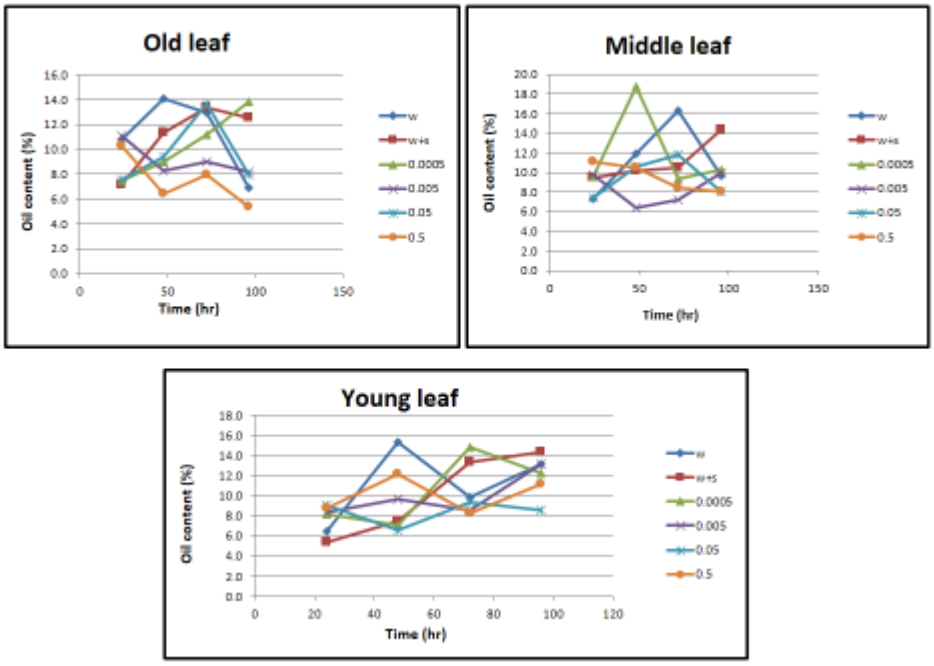

Figure 15. Leaf extraction results with chloroform/methanol solvent over developmental time. Extraction amounts are reported as the percent oil extracted from a dry weight of foliage of each plant. Each value represents the average of two different extraction samples made from leaves. W: water; W_S: water and surfactant; 0.5 mM of sodium oxamate; 0.05 mM of sodium oxamate; 0.005 mM of sodium oxamate; 0.0005 mM of sodium oxamate.

Table 1. The lipid content (%) in old leaves and concentration over time (hour), and six different concentration of sodium oxamate (water (w), water with surfactant (w+s), 0.0005mM, 0.005mM, 0.05mM, and 0.5mM).

	treatments					
time	Water	water and surfactant	0.0005mM Sodium oxamate	0.005mM Sodium oxamate	0.05mM Sodium oxamate	0.5mM Sodium oxamate
24 h	10.8	7.2	7.4	11.1	7.6	10.3
48 h	14.1	11.3	9.0	8.3	9.4	6.4
72 h	12.9	13.3	11.2	9.0	13.7	7.9
96 h	6.9	12.5	13.8	8.2	7.9	5.4

Table 2. The lipid content (%) in middle leaves and concentration over time (hour), and six different concentration of sodium oxamate (water (w), water with surfactant (w+s), 0.0005mM, 0.005mM, 0.05mM, and 0.5mM).

	treatments					
time	water	water and surfactant	0.0005mM Sodium oxamate	0.005mM Sodium oxamate	0.05mM Sodium oxamate	0.5mM Sodium oxamate
24 h	7.3	9.5	9.6	9.8	7.4	11.1
48 h	11.9	10.1	18.8	6.4	10.6	10.5
72 h	16.3	10.4	9.4	7.2	11.8	8.5
96 h	9.6	14.4	10.3	10.0	8.1	8.0

Table 3. The lipid content (%) in young leaves and concentration over time (hour), and six different concentration of sodium oxamate (water (w), water with surfactant (w+s), 0.0005mM, 0.005mM, 0.05mM, and 0.5mM.

	treatment					
time	water	water and surfactant	0.0005mM Sodium oxamate	0.005mM Sodium oxamate	0.05mM Sodium oxamate	0.5mM Sodium oxamate
24 h	6.4	5.4	8.1	8.4	9.1	8.7
48 h	15.3	7.5	7.0	9.6	6.6	12.1
72 h	9.9	13.3	14.8	8.6	9.4	8.3
96 h	13.1	14.3	12.3	13.1	8.6	11.2

Statistical analysis

A one-way ANOVA test was performed on leaf location and concentrations. All factors were fixed factors. The one-way ANOVA did not show leaf location and concentrations as statistically significant. A two-way ANOVA test was run on leaf location and concentrations to determine any interactions of these factors in affecting oil content. There was no significance for all interactions (Table 4).

Statistical analysis showed significant difference between time and the different concentration of sodium oxamate. Furthermore, even though leaf location and concentrations were independent variables, there were interactions of these factors affecting oil content. No significance was observed for leaf location because no oil body

was found in the cells. Sodium oxamate was found to affect *Schizochytrium sp*. by increasing oil body accumulation in its cells (Ashford et al., 2000). Tables 5 and 6 show mean for four different time intervals and six different levels of concentration of sodium oxamate, respectively.

Table 4: ANOVA on factors and their interactions that show F-value, which indicates that there is significant different. This result leads to reject null hypothesis.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Concentration	5	0.02467495	0.00493499	2.16	0.0630
Time	3	0.02244504	0.00748168	3.28	0.0235
Concentration*time	15	0.07140151	0.00476010	2.08	0.0150
Error	119	0.27181367	0.00228415		
Corrected Total	142	0.39020683			

CV = 31% , R^2 = 0.303411

Table 5: Mean for four different time intervals (24; 48; 72; and 96-hr) by Duncan test. 72 hour has highest mean among time.

Time (h)	Mean	Grouping
72 h	0.16333	A
96 h	0.15628	A
48 h	0.14839	A, B
24 h	0.12977	B

LSD=0.022

Table 6 : Mean for six different levels of concentration of sodium oxamate (water, water _ surfactant, 0.0005 mM, 0.005 mM, 0.05 mM, and 0.5 mM) by Duncan test. Water has highest mean among treatments.

Concentration	Mean	Grouping
Water	0.16825	A
Water _ Surfactant	0.16158	A
0.0005 mM sodium oxamate	0.15625	A, B
0.05 mM sodium oxamate	0.13865	A, B
0.005 mM sodium oxamate	0.13700	B
0.5 mM sodium oxamate	0.13529	B

Fisher's least significant difference = 0.0274

The interaction between time and concentration was statistically significant (P= 0.01) in effecting the amount of extracted lipid from leaves. Also, this interaction suggests that the amount of extracted lipid changes over time in each treated plant.

Thin layer chromatography (TLC)

The TLC results for lipids from each plant are shown in Figure 14. The bands for Fatty acids methyl ester, saturated fatty acids, tricylglycerol, sterol, and wax esters were identified.

TLC was used to understand the sectioning and extraction results. The main purpose of the TLC was to identify leaves lipid of different leaf location that treated by different concentration of sodium oxmate. Lin and Oliver (2008) found that 24 species are positive for oil bodies for the presence of TAGs. However, just 13 of the 24 species had a detectable level of TAGs. Also, Slocombe et al. (2009) reported that plants could partition fatty acids to TAG as a means to avoid the toxic effects of free fatty acids. Our results suggests that identified TAGs could be obtained from fatty acid in leaf. Moreover, TLC was used to study the composition of the extracted lipids. Also, the TLC showed lipids for monoglycerides, triglycerides, sterol, tocopherol, FAME, SAT, ST, and wax. Also, it is difficult to detect TAGs using TLC in all plants. However, they could be detected by GC-MS.

Kaup, Froese, and Thompson (2002) studied the lipid composition of leaves over the time in *Arabidopsis*. They found that in senescence leaves, the concentrations of DAGs, free fatty acids, TAGs, and wax esters were higher over polar lipids. This supports our result that lipid composition in leaves changes over the time.

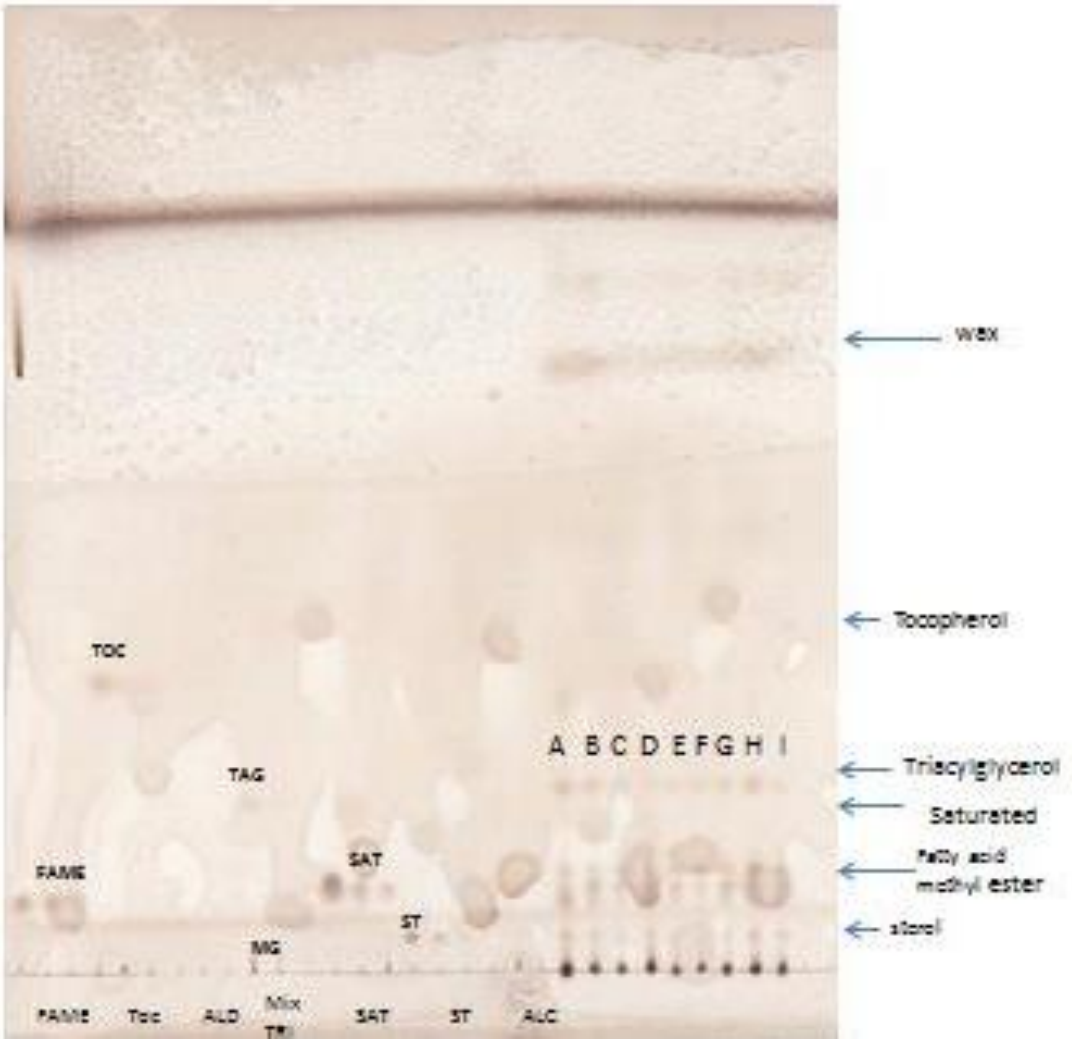

Figure 16. Thin layer chromatography (TLC) on standards and lipid from each treated with sodium oxamate plant plate shows FAME, SAT, sterols, and wax from samples. (A) 0.005 mM 48 h old, (B) water+surfactant 48 h young, (C) water+surfactant 48 h old, (D) water+surfactant 72 h young, (E) 0.05mM 48 h young, (F) 0.5 mM 48 h old, (G) water+surfactant 96 h young, (H) 0.0005 96 h old, and (I) 0.5 mM 96 h middle.

Table 7, 8 show quantification of leaf oil compounds in samples mg/g and percentage, respectively. Figure 17 shows standard curve for standard compounds in leaf lipids.

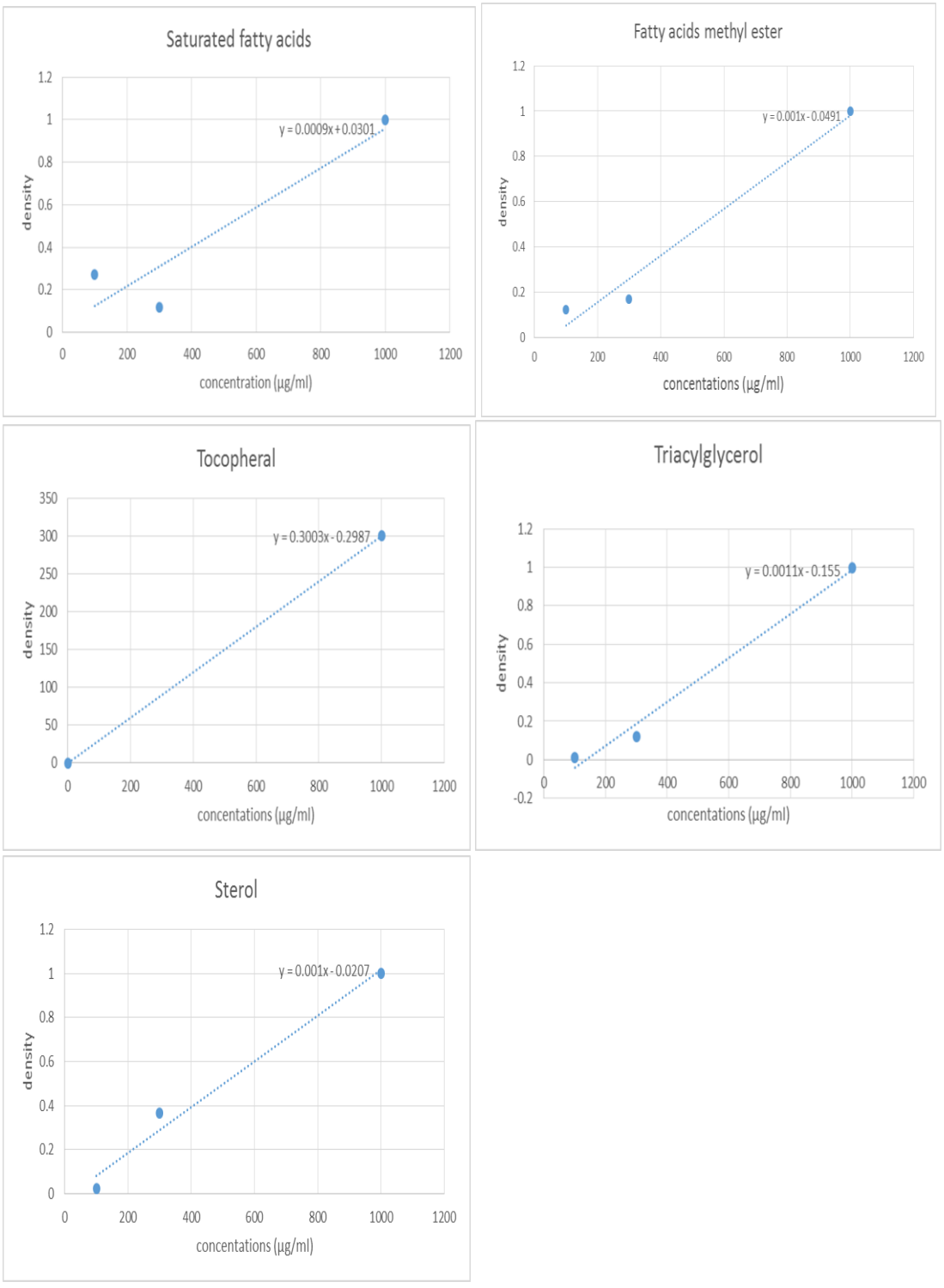

Figure 17. Standard curve for standard compounds in leaf lipids.

Table 7: Quantification of leaf oil compounds mg/g in samples.

	Leaf oil fraction				
Samples	Fatty acids methyl ester	Tocopheral	Triacylglycerol	saturated fatty acids	Sterol
0.005 mM 48hours old leaf	14.88	0.026	8.17	0.00059	11.60
W_S 48hours young leaf	32.23	0.039	15.67	0.00068	11.62
W_S 48hours old leaf	21.71	0.032	6.83	0.00066	3.23
W_S 72hours young leaf	17.40	0.029	5.25	0.00065	10.64
0.05 48hours young leaf	12.65	0.027	6.97	0.00065	4.58
0.5 48h old	2.88	0.022	3.34	0.00058	1.21
W_S 96hours young leaf	24.18	0.028	10.90	0.00076	5.50
0.0005 96hours old leaf	24.25	0.024	20.3	0.00067	13.87
0.5 96hours middle leaf	14.38	0.025	4.71	0.00058	7.75

Table 8: Quantification of leaf oil compounds (%) in samples.

	Leaf oil fraction				
Samples	Fatty acids methyl ester	Tocopheral	Triacylglycerol	saturated fatty acids	Sterol
0.005 mM 48hours old leaf	42.90	0.07	23.56	0.001701	33.44
W_S 48hours young leaf	54.11	0.06	26.30	0.001141	19.51
W_S 48hours old leaf	68.25	0.10	21.48	0.002074	10.15
W_S 72hours young leaf	52.20	0.08	15.77	0.00195	31.93
0.05 48hours young leaf	52.20	0.11	28.78	0.002681	18.89
0.5 48hours old leaf	38.66	0.29	44.77	0.007762	16.25
W_S 96hours young leaf	59.52	0.07	26.84	0.001871	13.55
0.0005 96hours old leaf	41.49	0.04	34.72	0.001146	23.73
0.5 96hours middle leaf	53.51	0.09	17.54	0.002158	28.84

CHAPTER V

CONCLUSIONS

This study is a first step for developing the use of sodium oxamate that could lead to improved biofuel production. Also, this study deals with various topics such as plant biochemistry, plant cell physiology, leaf lipid composition, and others.

Regarding the result of this study and literatures in the discussion sections, great conclusions can be derived from these experiments. From the sectioning results, no oil bodies were observed in leaf mesophyll cells in this study. The primary conclusion from the extraction results is that a small concentration of sodium oxamate could increase leaf lipids. The primary conclusion from the TLC analysis is that fatty acids could be partitioned to TAGs.

Moreover, in this study the *Brassica napus* cultivar did not contain oil bodies in plant mesophyll cells; however, it may contain FAME, tocopherol, sterol, triaclyglycerol, and saturated fatty acids. The question of how sodium oxamate affects oil content in organisms can be answered with further studies, such as biochemistry and physiology, that are employed to understand the role of sodium oxamate on oil content. Additionally, sodium oxamate should be tested on oil bodies in the leaves, seeds, and stems of other plant to understand its effects.

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